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14. ABSTRACT
Recombinant antibody and DNA technologies have been used to improve the sensitivity and specificity of rapid assays for the detection of B. anthracis spores. We have identified a spore coat gene, cotK, which demonstrates substantial sequence variation among B. anthracis clade members including B. cereus, B. thuringiensis and B. mycoides strains. A central 25 amino acid region of the CotK protein was identified that varies in sequence among the B. anthracis clade strains analyzed. Peptides were synthesized containing the 25 residue CotK variable region from B. cereus, B. thuringiensis and B. anthracis strains. These peptides, when coupled with a carrier conjugate, were shown to produce antibody responses in animal models.

15. SUBJECT TERMS
Anthrax, spore coat, antibodies, genetic variation

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FINAL REPORT

Grant Number: N00014-99-1-0268

Principal Investigator: Terrance Leighton

Institution: University of California Berkeley

Grant Title: Recombinant Antibodies Specific to *Bacillus anthracis* Spores

Award Period: 1/1/99 - 12/30/00

OBJECTIVE: The following questions are being addressed:

1. How can advanced recombinant DNA and antibody technology be exploited to improve the specificity of *Bacillus anthracis* rapid detection devices?
2. How can advanced recombinant DNA and antibody technology be exploited to improve the sensitivity of *Bacillus anthracis* rapid detection devices?
3. How can anthrax detector reagent consistency and reproducibility be improved?

APPROACH: Recently, methods been have developed for the generation of complementary DNA (cDNA) libraries of antibody encoding immunoglobulin genes and expression of these genes in production hosts such as the bacterium *Escherichia coli*. For this very promising technology to be applied successfully to the rapid detection of *B. anthracis*, large quantities of pure anthrax-specific antigens are required. These antigens must be displayed on infectious anthrax spores. Presently, very little is known about the structure and genetic specification of *B. anthracis* spore coat surface signatures.

Rapid assays based on anthrax spore coat components have the following advantages:

1. Spore structural components (spore coat and small acid soluble spore core proteins) account for over 50% of the total spore protein content.
2. Analogous proteins only exist in endospore forming bacteria.
3. There are no structural counterparts produced in vegetative cells.
4. Spore structural proteins are essential for spore resistance to heat, chemicals, UV, enzymes and mechanical disruption.

Spore coat genes of particular interest are those which encode components of the outer spore surface. Several spore coat proteins may be conserved throughout the *Bacillus* group. If this is the case, antibodies directed against these determinants will likely be cross-reactive with both near- and distant-neighbors. This situation could explain the lack of specificity observed previously with antibodies produced against whole anthrax spores. To alleviate this problem, we have focused on isolating and characterizing spore coat genes with DNA sequences specific for *B. anthracis*.

ACCOMPLISHMENTS:

In this report we focus on the application of the outer spore coat gene *cotK* as an alternative or complimentary tool for developing recombinant antibody technology to identify members of the *B. anthracis* clade.

DNA sequence analysis of *B. anthracis* group *cotK* amplicons has allowed a phylogenetic separation of the strains examined into protein sequence clusters (Proteotypes). Analysis of further strains from the *B. anthracis* group may reveal additional CotK Proteotypes.

The initial results obtained from *B. anthracis* clade *cotK* DNA sequence analysis suggest that there is significant genetic variation among the *B. cereus*, *B. thuringiensis* and *B. anthracis* strains analyzed. These data also suggest that the genetic clustering of *B. anthracis* clade strains based on *cotK* signatures is different from the clustering observed with a spore core protein gene. It is possible that outer spore coat genes may be subjected to additional evolutionary constraints that do not operate on spore core genes. These results also suggest that the DNA sequence analysis of outer spore coat genes may be of considerable value in selecting protein and epitope targets for spore-specific immunoassay development. This experimental strategy can also be applied to other *B. anthracis* spore coat proteins.

NMRC and other laboratories have employed synthetic peptides synthesized from regions of CotK that contain the most variable sequence motifs. We have been able to produce polyclonal antibodies to these signature region peptides. Preliminary results suggest that the specificity and sensitivity of these antibody reagents is promising.

CONCLUSIONS: These results suggest that phylogenetic DNA sequence analysis of outer spore coat genes may provide a new paradigm for rapidly selecting protein epitope targets for spore-specific immunoassay development. Previously, identification of protein motifs that determined epitopic specificity was a tedious process involving random scanning of protein sequence space to identify informative signatures. Using the phylogenetic analysis methods described here, emergent sequence properties associated with the natural genetic history of the protein of interest reveal epitopic targets of opportunity.

SIGNIFICANCE: These studies establish that CotK spore coat protein sequences possess sufficient genetic variation to be a promising target for developing recombinant antibodies that recognize *B. anthracis* spores.

PATENT INFORMATION: No patents have been filed

AWARD INFORMATION: Dr. El Helow was awarded a Fulbright Faculty fellowship

PUBLICATIONS and ABSTRACTS (for total period of grant):

1. Longchamp, P. and T. Leighton. 1999. Molecular recognition specificity of *Bacillus anthracis* spore antibodies. J. Appl. Microbiol., 87: 246.

2. Longchamp, P. and T. Leighton. 2000. Molecular recognition specificity of *Bacillus globigii* spore antibodies. Letters in Applied Microbiology, 31:242.

3. Leighton, T. 1999. Biology/Germination of *B. anthracis*. DARPA DSRC Decon '99, Workshop 1: Underlying Biology and Environmental Stability of BW Agents, Current Decontamination Technologies, Arlington, Virginia.

4. Leighton, T. 1999. Biological Targets of Opportunity. DARPA DSRC Decon '99, Workshop 2: Advanced Decontamination Technologies, Logistics of Decontamination for Vehicles and Buildings, Arlington, Virginia.

5. Leighton, T. 1999. "Reagent Grade" Surrogates and Quantitative Test Protocols for Decon Agents/Techniques. DARPA DSRC Decon '99 Workshop 3: Advanced Decontamination Technologies, Logistics of Decontamination for Vehicles and Buildings, Arlington, Virginia.

6. Leighton, T. 1999. Anthrax (Example Decon-Resistant Organism) - Review of Biology and Special Opportunities for Decontamination. Decon Study Briefing, DARPA DSRC Summer Conference, La Jolla, California.

7. Eisenstadt, E., Reid, T., Peterson, S., Baillie, L., McKinney, N., Leighton, T., and J. Hunter-Cevera. 1999. Whole-Genome Sequencing of *Bacillus anthracis*. 1999. DP99, 1st European Conference on Dangerous Pathogens, Winchester, England.

8. Leighton, T. 2000. Anthrax: solving Problems with Genomic Information. The Challenges of Infectious disease in the 21st Century, Banbury Conference, Cold Spring Harbor, New York.

9. Leighton, T. 2000. *Ab initio* Construction of Definitive Anthrax Detection reagents. The Challenges of Infectious disease in the 21st Century, Banbury Conference, Cold Spring Harbor, New York.

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Molecular recognition specificity of *Bacillus anthracis* spore antibodies

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P. LONGCHAMP AND T. LEIGHTON. 1999. The sensitivity and specificity of polyclonal and monoclonal antibodies raised against anthrax spore preparations has been assessed by Western blotting. None of the antibodies studied were completely specific in recognizing the anthrax spore surface. A polyclonal serum recognized a wide range of spore surface epitopes and demonstrated limited cross-reaction with the near-neighbour species *Bacillus cereus* spore surface. Two monoclonal antibodies studied demonstrated more extensive cross-reaction with distant-neighbour species *B. globigii* and *B. subtilis*. These monoclonal antibodies did not react with spore surface epitopes but did bind strongly to vegetative cell epitopes in all four *Bacillus* species studied.

INTRODUCTION

Immunoassays are the current foundation for many rapid pathogen detection technologies. Existing antibody reagents for the identification of *Bacillus anthracis* spores may, however, be limited in their ability to distinguish *B. anthracis* from environmentally ubiquitous near- and distant-neighbour species such as *B. cereus*, *B. subtilis* and *B. globigii* (Phillips *et al.* 1988; Titball *et al.* 1991). Developing species-specific anthrax immunoassays requires the production of specific reagents, i.e. polyclonal or monoclonal antibodies that recognize structures that are unique to the target organism and which distinguish it from its commonly occurring near- and distant-neighbour species. We have utilized Western blotting methods to analyse a suite of existing monoclonal and polyclonal antibodies produced against *B. anthracis* spore antigens to assess the species and molecular target recognition specificity of these reagents. Epitopic specificity and molecular recognition reactions were analysed for cytoplasmic and surface extracts from *B. anthracis*, *B. cereus*, *B. globigii* and *B. subtilis* spores and vegetative cells.

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MATERIALS AND METHODS

Strains

The following strains were used: *B. anthracis UM23, ura* (Thorne, University of Massachusetts, Amherst, MA); *B. cereus T* (Bacillus Genetic Stock Centre, Columbus, Ohio, USA); *B. globigii* (this laboratory), and *B. subtilis* 168 *trpC2* (this laboratory).

Polyclonal and monoclonal antibodies

AB-BaN. Obtained from Dr A. Churilla (Naval Medical Research Institute, Bethesda, MD, USA). Rabbit anti-*B. anthracis* 070497–02 polyclonal antibody (2·3 $\mu g\ \mu l^{-1}$ in 50% glycerol) produced by a regimen of spore immunization followed by vegetative cell immunization with a mixture of *B. anthracis* strains.

MAB-Ba1B7 and MAB-Ba2B7. Obtained from Dr A. Churilla. Monoclonal mouse anti-*B. anthracis* antibodies (prepared by Dr T. Webber (CBD DERA, Porton Down, UK)) produced using *B. anthracis* (Vollum) spore preparations. The concentrations of these antibodies were 2·5 and 2·75 $\mu g\ \mu l^{-1}$ (50% glycerol), respectively.

Cell growth, sporulation and protein methods

Vegetative cells and spores were prepared in 2 × SG medium as described by Leighton and Doi (1971). Ballistic disruption

(0·1 mm glass beads) of spores and vegetative cells was achieved during a 4 min treatment at homogenizing speed in a Biospec Products Bead Beater (Biospec Products, Bantlesville, Oklahoma). Cell and spore surface extracts were prepared as described by Aronson and Fitz-James (1976). Vegetative and spore protein fractions were prepared for electrophoretic analysis in an extraction buffer containing 0·12 mol l^{-1} Tris base, 4% (w/v) SDS, 1 mol l^{-1} β-mercaptoethanol, 20% (w/v) glycerol and 0·002% (w/v) bromophenol blue. Lyophilized spore (5 mg dry weight) and 10–20 mg (dry weight) of mid-log phase vegetative cell fractions were resuspended in 0·5 ml of extraction buffer. The samples were boiled for 3 min and centrifuged for 6 min at 14 000 rev min^{-1}. The upper 250 μl of the extract were removed and a constant protein concentration from each fraction was loaded (approximately 15 μl) onto a 4–12% NuPAGE MES gel (Novex, San Diego, California). Electrophoretic separation was performed according to the manufacturer's recommendation (Novex).

Western blot methods

The ECL Western blot system (Amersham, Pharmacia Biotech, Piscataway, NJ) was used to visualize proteins detected by horseradish peroxidase-labelled secondary antibodies. Proteins separated on 4–12% NuPAGE gels were transferred to nitro-cellulose membranes by semi-dry transfer blotting (Bio-Rad, Hercules, California). A 1/5000 dilution of primary and secondary antibodies was used during the immunodetection step for monoclonal antibodies and a 1/10 000 dilution of primary and secondary antibodies was used during the immunodetection step for polyclonal antibodies.

RESULTS

We have examined the molecular recognition specificity of the *B. anthracis* polyclonal AB-BaN antibody with vegetative and spore subcellular fractions derived from *B. globigii*, *B. subtilis*, *B. cereus* and *B. anthracis* (Fig. 1). The AB-BaN recognition specificity was restricted primarily to the anthrax group, as *B. globigii* and *B. subtilis* protein fractions did not cross-react strongly. This polyclonal antibody recognized a large number of anthrax spore surface epitopes and had limited cross-reaction with the near-neighbour *B. cereus* spore surface (Fig. 1a). A high degree of anthrax immunospecificity was also seen among the epitopes recognized in vegetative cell extracts (Fig. 1b).

Two monoclonal antibodies, MAB-Ba1B7 and MAB-Ba2B7 raised against *B. anthracis* spore preparations, were analysed for their molecular recognition specificity (Fig. 2). The MAB-Ba1B7 monoclonal antibody (Fig. 2a, b) reacted strongly with one protein of approximately 95 kDa in *B. anthracis* vegetative cells and more weakly with a similar epitope in spore cytoplasmic extracts. This monoclonal antibody did not detect epitopes on spore surfaces. The MAB-Ba1B7 antibody also cross-reacted with an epitope of similar molecular weight in the cytoplasmic and cell surface extracts of the near-neighbour species *B. cereus* and

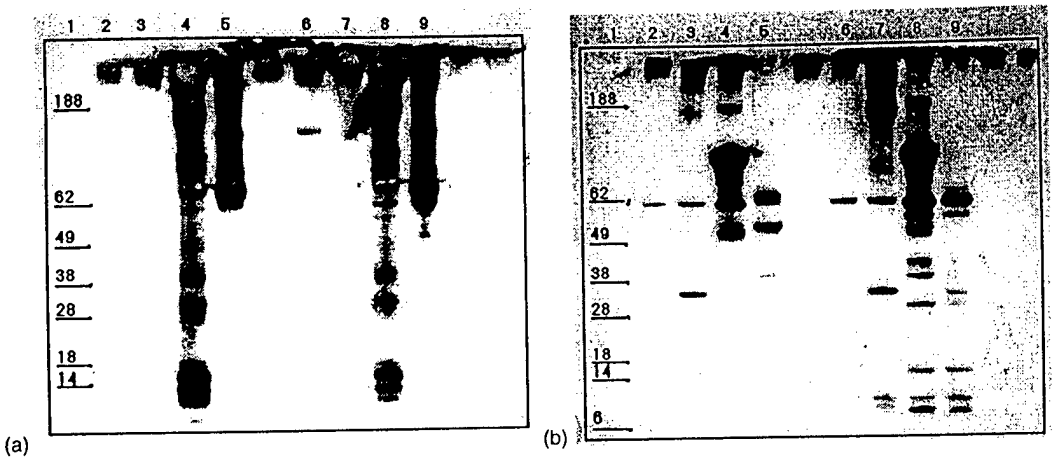

Fig. 1 Western blot analysis of *Bacillus anthracis* AB-BaN polyclonal antibody molecular recognition specificity. (a) Spore extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* spore surface extract; 3, *B. subtilis* spore surface extract; 4, *B. anthracis* spore surface extract; 5, *B. cereus* spore surface extract; 6, *B. globigii* spore cytoplasm extract; 7, *B. subtilis* spore cytoplasm extract; 8, *B. anthracis* spore cytoplasm extract; 9, *B. cereus* spore cytoplasm extract. (b) Vegetative cell extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* cell surface extract; 3, *B. subtilis* cell surface extract; 4, *B. anthracis* cell surface extract; 5, *B. cereus* cell surface extract; 6, *B. globigii* cell cytoplasm extract; 7, *B. subtilis* cell cytoplasm extract; 8, *B. anthracis* cell cytoplasm extract; 9, *B. cereus* cell cytoplasm extract

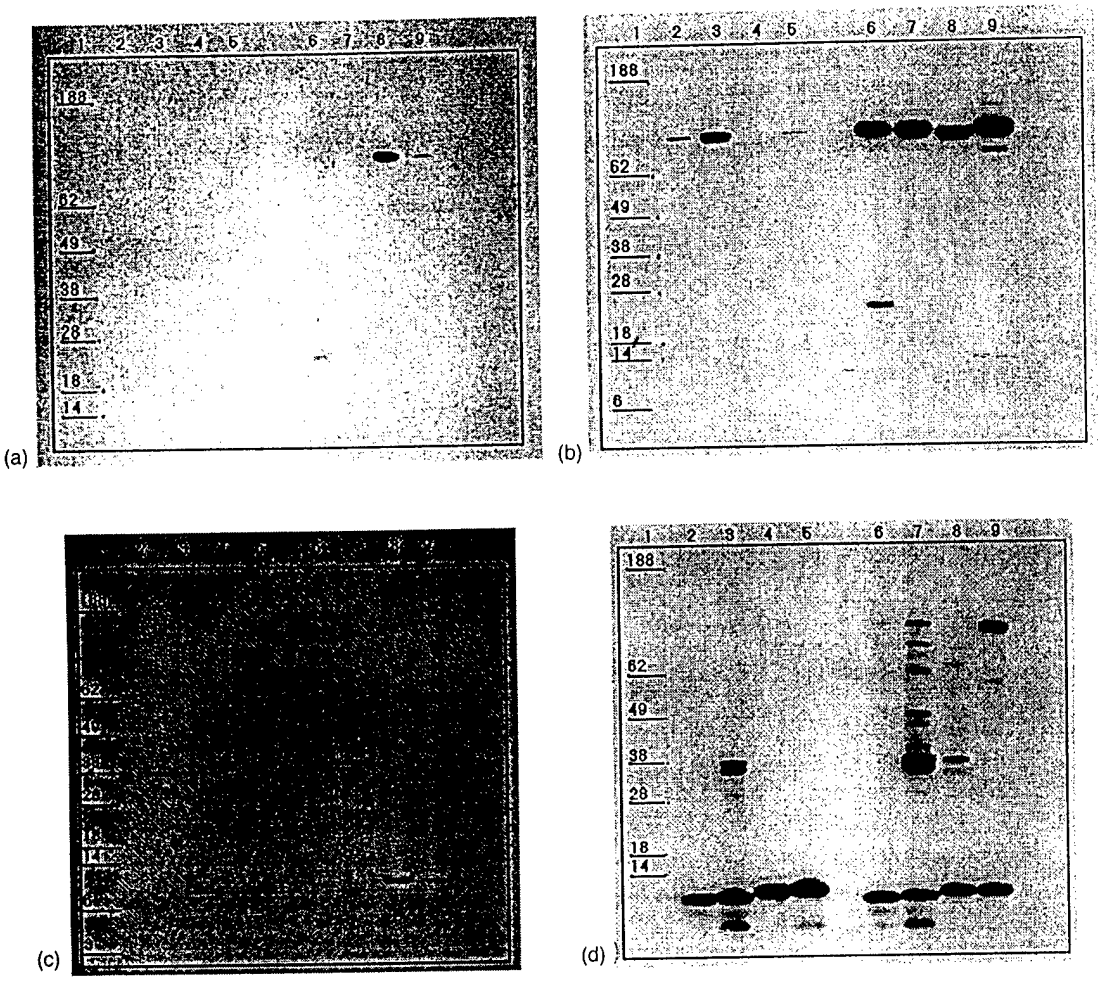

Fig. 2 Western blot analysis of *Bacillus anthracis* MAB-Ba1B7 (a and b) and MAB-Ba2B7 (c and d) monoclonal antibody molecular recognition specificity. (a) and (c) contain spore extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* spore surface extract; 3, *B. subtilis* spore surface extract; 4, *B. anthracis* spore surface extract; 5, *B. cereus* spore surface extract; 6, *B. globigii* spore cytoplasm extract; 7, *B. subtilis* spore cytoplasm extract; 8, *B. anthracis* spore cytoplasm extract; 9, *B. cereus* spore cytoplasm extract. (b) and (d) contain vegetative cell extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* cell surface extract; 3, *B. subtilis* cell surface extract; 4, *B. anthracis* cell surface extract; 5, *B. cereus* cell surface extract; 6, *B. globigii* cell cytoplasm extract; 7, *B. subtilis* cell cytoplasm extract; 8, *B. anthracis* cell cytoplasm extract; 9, *B. cereus* cell cytoplasm extract

the distant-neighbour species *B. subtilis* and *B. globigii*. The MAB-Ba2B7 monoclonal antibody (Fig. 2c, d) reacted strongly with one protein of approximately 10 kDa in *B. anthracis* vegetative cells and more weakly with a similar epitope in spore cytoplasmic extracts. This monoclonal antibody did not detect epitopes on spore surfaces. The MAB-Ba2B7 antibody also cross-reacted strongly with an epitope of similar molecular weight in the cytoplasmic and cell surface extracts of the four *Bacillus* species examined. This monoclonal antibody did not detect epitopes on spore surfaces.

DISCUSSION

The ability to rapidly and specifically detect the anthrax spore, the most persistent and dispersible form of *B. anthracis*, is of great importance to disease control (Titball *et al.* 1991). Anthrax spore immunochemical assays are one of the most attractive technologies for the rapid and inexpensive detection of *B. anthracis* in medical and environmental samples. The purpose of this study was to assess the molecular recognition specificity of several existing anthrax spore antibody preparations to guide the development of assays with improved specificity and sensitivity. Several questions regarding anti-

body sensitivity and specificity were addressed. Is the anthrax spore surface immunogenic? What is the nature and distribution of epitopes recognized by antibodies raised against spore preparations? Are the recognized epitopes exclusively found in spores, or are they also present in vegetative cells? Do these antibodies cross-react with epitopes from near- and distant-neighbour *Bacillus* species? Do monoclonal antibodies afford improved specificity when compared with polyclonal antibodies?

The data shown in Fig. 1 demonstrate that a polyclonal antibody preparation (AB-BaN) raised against anthrax spore and vegetative cell antigens recognized a diverse range of anthrax spore epitopes. These data demonstrate that the anthrax spore surface is immunogenic and that antibodies can be generated against a variety of spore surface proteins. The Ab-BaN antibody cross-reacts with a limited number of epitopes on the near-neighbour *B. cereus* spore surface but does not react with the distant-neighbour *B. globigii* and *B. subtilis* spore surfaces.

The data presented in Fig. 2 demonstrate that monoclonal antibody preparations (MAB-Ba1B7 and MAB-Ba2B7) raised against anthrax spore preparations had a more narrow range of epitopic response, but a diminished recognition specificity when compared with the polyclonal antibody. Both monoclonal antibodies recognized epitopes present in near- and distant-neighbour *Bacillus* species to anthrax. Although these antibodies were generated with anthrax spore preparations, their primary avidity was directed against vegetative cell epitopes. The MAB-Ba1B7 and MAB-Ba2B7 monoclonal antibodies did not recognize spore surface epitopes. These results suggest that spore preparations, or isolated spore surface antigens, of higher purity may be required to develop monoclonal antibodies that recognize the anthrax spore surface specifically.

The data presented demonstrate that it is possible to generate antibodies that recognize the anthrax spore surface. The development of anthrax-specific polyclonal or monoclonal antibodies will require the isolation and purification of species-specific spore surface antigens. These studies are in progress in our laboratory and elsewhere.

ACKNOWLEDGEMENTS

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Phillips, A.P., Campbell, A.M. and Quinn, R. (1988) Monoclonal antibodies against spore antigens of *Bacillus anthracis*. *FEMS Microbiology Immunology* **47**, 169–178.

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Molecular recognition specificity of *Bacillus globigii* spore antibodies

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P. LONGCHAMP AND T. LEIGHTON. 2000. Western blotting methods have been used to assess the specificity of polyclonal antibodies raised against *Bacillus globigii* spore and vegetative cell preparations. None of the antibodies studied were completely species-specific in their recognition of spore surface epitopes. One polyclonal serum recognized several spore surface epitopes and demonstrated limited cross-reaction with the spore surface of the near-neighbour species *B. subtilis*. A second polyclonal serum, raised against aged spore antigens, recognized damaged spore epitopes primarily. Both of these antibodies also cross-reacted with vegetative cell epitopes present in all four *Bacillus* species (*B. globigii, B. subtilis, B. cereus and B. anthracis*) studied.

INTRODUCTION

Bacillus globigii spores have been used extensively as a tracer in environmental fate and transport studies (Casewell *et al.* 1984; Houston *et al.* 1989; Horan *et al.* 1991). Antibody-based assays for *B. globigii* spores are frequently employed to monitor the distribution of this tracer organism in environmental sample matrices. These reagents may, however, be limited in their ability to distinguish *B. globigii* from environmentally ubiquitous near- and distant-neighbour species such as *B. subtilis and B. cereus* (Quinlan and Foegeding 1997; Phillips *et al.* 1988; Titball *et al.* 1991). ELISA analysis of *B. globigii* polyclonal antibody specificity (Churilla, personal communication) demonstrated cross-reaction with *B. subtilis*, *B. cereus* and *B. thuringiensis* cell and spore samples. In order to better understand the molecular interactions of *B. globigii* polyclonal antibodies with cytoplasmic and surface antigens we have utilized Western blotting methods to examined the epitopic specificity of these sera for extracts prepared from *B. globigii*, *B. subtilis*, *B. cereus* and *B. anthracis* spores and vegetative cells.

MATERIALS AND METHODS

The strains used throughout these studies are shown in Table 1.

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Polyclonal antibodies

AB-BgN. This was obtained from Dr A. Churilla (Naval Medical Research Institute). Rabbit anti-*B. globigii* 270696-01 polyclonal antibody was produced following an immunization regimen using a mixture of vegetative cell and spore antigens (3·5 $\mu g\ \mu l^{-1}$ in 50% glycerol).

AB-BgD. This was obtained from Dr D. Martin (Dugway Proving Ground). Goat anti-*B. globigii* polyclonal antibody was produced following an immunization regimen using a mixture of aged spore and vegetative cell antigens (3·5 $\mu g\ \mu l^{-1}$ in 50% glycerol).

Cell growth, sporulation and protein methods

Vegetative cells and spores were prepared in 2×SG medium as described by Leighton and Doi (1971). Ballistic disruption (0·1-mm glass beads) of spores and vegetative cells was achieved during a 4-min treatment at homogenizing

Table 1 Strains and their source

Strains	Source
Bacillus anthracis UM23, ura	Thorne
B. cereus T	Bacillus Genetic Stock Center
B. globigii	This laboratory
B. subtilis 168 *trpC2*	This laboratory

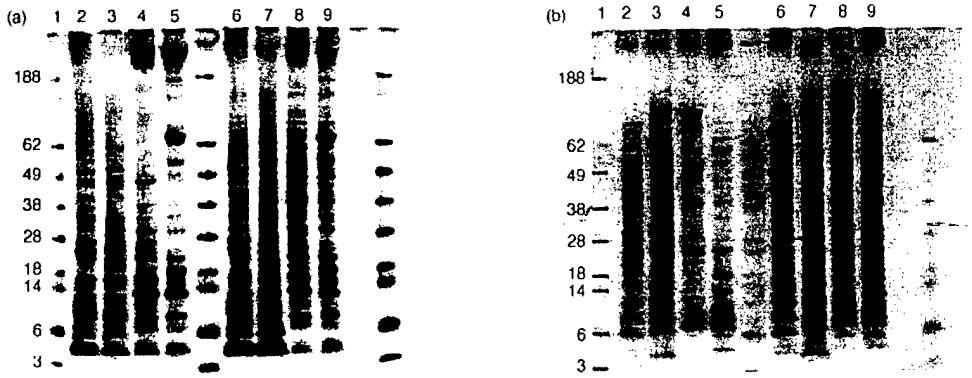

Fig. 1 (a) Spore extracts. Lanes: 1, molecular weight standards; 2, *Bacillus globigii* spore surface extract; 3, *B. subtilis* spore surface extract; 4, *B. anthracis* spore surface extract; 5, *B. cereus* spore surface extract; 6, *B. globigii* spore cytoplasm extract; 7, *B. subtilis* spore cytoplasm extract; 8, *B. anthracis* spore cytoplasm extract; 9, *B. cereus* spore cytoplasm extract. (b) Vegetative cell extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* cell surface extract; 3, *B. subtilis* cell surface extract; 4, *B. anthracis* cell surface extract; 5, *B. cereus* cell surface extract; 6, *B. globigii* cell cytoplasm extract; 7, *B. subtilis* cell cytoplasm extract; 8, *B. anthracis* cell cytoplasm extract; 9, *B. cereus* cell cytoplasm extract

speed in a Bead Beater (Biospec Products). Cell and spore surface extracts were prepared as described by Aronson and Fitz-James (1976). Vegetative and spore protein fractions were prepared for electrophoretic analysis in an extraction buffer containing 0·12 mol l^{-1} Tris-base, 4% (w/v) sodium dodecyl sulphate, 1 mol l^{-1} β-mercaptoethanol, 20% (w/v) glycerol and 0·002% (w/v) bromophenol blue. Five mg (dry weight) lyophilized spore and 10–20 mg

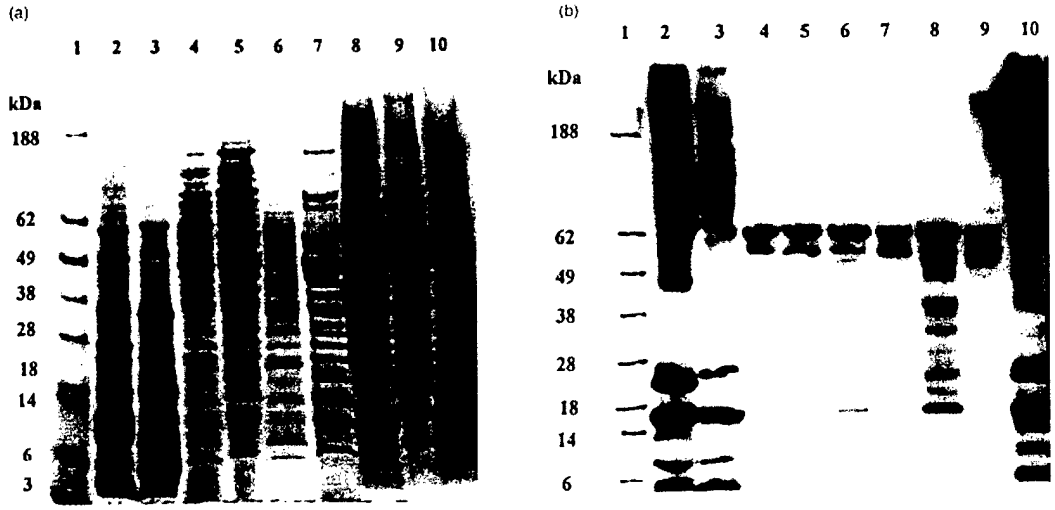

Fig. 2 (a) PAGE separation of purified *Bacillus globigii* and *B. subtilis* subcellular fractions. Lanes: 1, molecular weight standards; 2, *B. globigii* purified spore cytoplasm extract; 3, *B. subtilis* purified spore cytoplasm extract; 4, *B. globigii* vegetative cell cytoplasm extract; 5, *B. subtilis* vegetative cell cytoplasm extract; 6, *B. globigii* cell wall extract; 7, *B. subtilis* cell wall extract; 8, *B. globigii* membrane extract; 9, *B. subtilis* membrane extract; 10, *B. globigii* aged spore surface extract. (b) Western blot analysis of *B. globigii* AB-BgD polyclonal antibody molecular recognition specificity. Lanes: 1, molecular weight standards; 2, *B. globigii* purified spore cytoplasm extract; 3, *B. subtilis* purified spore cytoplasm extract; 4, *B. globigii* vegetative cell cytoplasm extract; 5, *B. subtilis* vegetative cell cytoplasm extract; 6, *B. globigii* cell wall extract; 7, *B. subtilis* cell wall extract; 8, *B. globigii* membrane extract; 9, *B. subtilis* membrane extract; 10, *B. globigii* aged spore surface extract

(dry weight) mid-log phase vegetative cell fractions were resuspended in 0·5 ml extraction buffer. The samples were boiled for 3 min and centrifuged for 6 min at 14 000 rev min^{-1}. The upper 250 μl of the extract was removed and a constant protein concentration from each fraction was loaded (approximately 15 μl) onto a 4–12% NuPAGE MES gel (Novex). Electrophoretic separation was performed according to the manufacturer's recommendations.

Western blot methods

The ECL Western blot system (Amersham) was used to visualize proteins detected by horseradish peroxidase-labelled secondary antibodies. Proteins separated on 4–12% NuPAGE gels were transferred to nitrocellulose membranes by semi-dry transfer blotting (Bio-Rad). A 1/10 000 dilution of primary and secondary antibodies was used during the immunodetection step.

RESULTS

Figure 1 illustrates the separation of *B. globigii, B. subtilis, B. anthracis* and *B. cereus* vegetative and spore subcellular protein fractions achieved with the described polyacrylamide gel electrophoresis system. Figure 2 depicts the reaction of AB-BgD *B. globigii* antibodies with vegetative and spore subcellular protein fractions derived from *B. globigii* and the near-neighbour species *B. subtilis*. Extensive AB-BgD antibody cross-reaction occurred with a wide range of *B. subtilis* subcellular fractions. In addition, the primary reaction of this polyclonal serum with spore-derived fractions appeared to be directed against intracellular epitopes (Fig. 2b, lanes 2 and 10). These results (Fig. 2) suggested that AB-BgD recognized epitopes primarily derived from damaged or germinated spores. The data presented in Fig. 3 confirmed this inference by demonstrating that AB-BgD did not react strongly with spore surface epitopes derived from highly purified *B. globigii* and *B. subtilis* spore preparations. AB-BgD did, however, react strongly with similar extracts prepared from aged *B. globigii* spore preparations (the immunizing antigen). Further experiments were performed (Fig. 4) to explore the reaction of AB-BgD and AB-BgN antibodies with vegetative and spore subcellular protein fractions derived from *B. globigii*, the near-neighbour species *B. subtilis* and the distant-neighbour species *B. cereus* and *B. anthracis*.

The results obtained with the AB-BgD antibody (Fig. 4a,b) are consistent with the data shown in Fig. 3 and confirm that this antibody recognized damaged spore epitopes derived primarily from the spore cytoplasm. The AB-BgD recognition specificity for damaged spore epitopes was limited to the near-neighbour species *B. globigii* and *B. subtilis*. This antibody also recognized vegetative cell epitopes

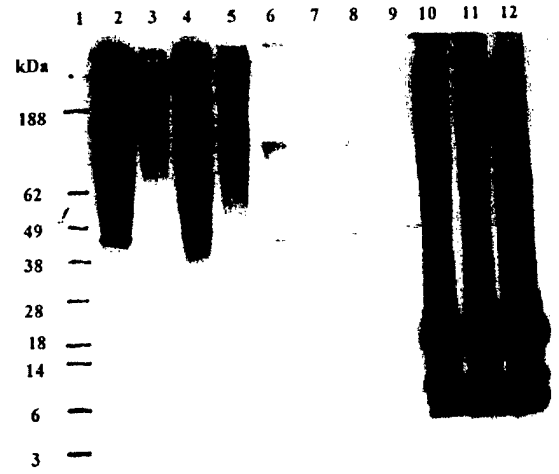

Fig. 3 Western blot analysis of AB-BgD reaction with *Bacillus globigii* and *B. subtilis* spore surface extracts. Lanes: 1, molecular weight standards; 2, purified *B. globigii* spores (Tris pH 8·0, 500 mmol l^{-1} NaCl extract); 3, purified *B. globigii* spores (Tris pH 8·0, 500 mmol l^{-1} NaCl, 8 mol l^{-1} urea extract); 4, purified *B. globigii* spores (0·1 mol l^{-1} NaOH extract); 5, purified *B. globigii* spores (5% acetic acid extract); 6, purified *B. globigii* spores (Tris pH 8·0 extract); 7, purified *B. subtilis* spores (Tris pH 8·0, 500 mmol l^{-1} NaCl, 8 mol l^{-1} urea extract); 8, purified *B. subtilis* spores (0·1 mol l^{-1} NaOH extract); 9, purified *B. subtilis* spores (5% acetic acid extract); 10, aged *B.globigii* spores (Tris pH 8·0, 500 mmol l^{-1} NaCl extract); 11, aged *B. globigii* spores (Tris pH 8·0, 500 mmol l^{-1} NaCl, 8 mol l^{-1} urea extract); 12, aged *B. globigii* spores (0·1 mol l^{-1}NaOH extract)

found in all of the *Bacillus* species examined. The AB-BgN antibody (Fig. 4c,d) recognized *B. globigii* spore surface epitopes. There was limited cross-reaction with *B. subtilis* spore surface epitopes. It is not clear whether the spore surface and cytoplasm epitopes recognized by this antibody were identical. No cross-reaction was seen with spore surface extracts from the distant-neighbour species *B. cereus* and *B. anthracis*. AB-BgN recognized several vegetative cell epitopes that were present in both cell surface and cell cytoplasm extracts. A number of these surface and cytoplasmic epitopes were conserved in all four *Bacillus* species examined.

DISCUSSION

The ability to detect *B. globigii* spores rapidly and specifically is of considerable interest for environmental tracer studies (Casewell *et al.* 1984; Houston *et al.* 1989; Horan *et al.* 1991). Spore immunochemical assays are one of the

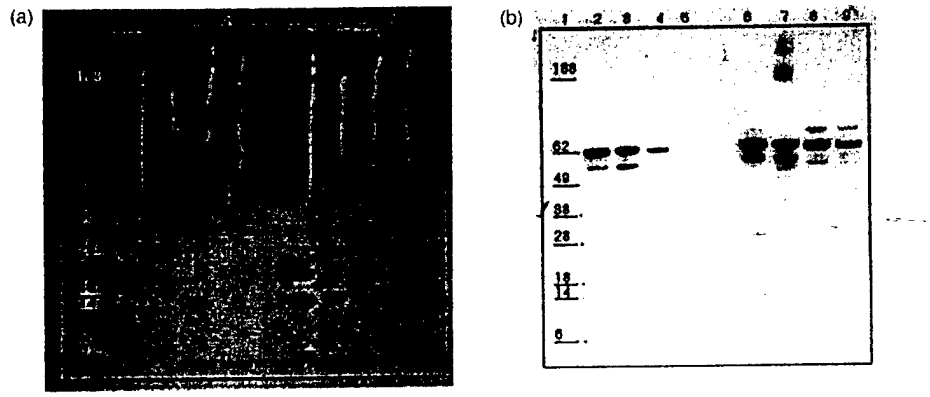

Fig. 4 Western blot analysis of *Bacillus globigii* AB-BgD polyclonal antibody molecular recognition specificity. (a) Spore extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* spore surface extract; 3, *B. subtilis* spore surface extract; 4, *B. anthracis* spore surface extract; 5, *B. cereus* spore surface extract; 6, *B. globigii* spore cytoplasm extract; 7, *B. subtilis* spore cytoplasm extract; 8, *B. anthracis* spore cytoplasm extract; 9, *B. cereus* spore cytoplasm extract. (b) Vegetative cell extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* cell surface extract; 3, *B. subtilis* cell surface extract; 4, *B. anthracis* cell surface extract; 5, *B. cereus* cell surface extract; 6, *B. globigii* cell cytoplasm extract; 7, *B. subtilis* cell cytoplasm extract; 8, *B. anthracis* cell cytoplasm extract; 9, *B. cereus* cell cytoplasm extract. (c) Spore extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* spore surface extract; 3, *B. subtilis* spore surface extract; 4, *B. anthracis* spore surface extract; 5, *B. cereus* spore surface extract; 6, *B. globigii* spore cytoplasm extract; 7, *B. subtilis* spore cytoplasm extract; 8, *B. anthracis* spore cytoplasm extract; 9, *B. cereus* spore cytoplasm extract. (d) Vegetative cell extracts. Lanes: 1, molecular weight standards; 2, *B. globigii* cell surface extract; 3, *B. subtilis* cell surface extract; 4, *B. anthracis* cell surface extract; 5, *B. cereus* cell surface extract; 6, *B. globigii* cell cytoplasm extract; 7, *B. subtilis* cell cytoplasm extract; 8, *B. anthracis* cell cytoplasm extract; 9, *B. cereus* cell cytoplasm extract

most attractive technologies for detecting the presence of *B. globigii* spores in environmental matrices. The purpose of this study was to assess the molecular recognition specificity of two existing spore antibody preparations to guide the development of assays with improved specificity and sensitivity. Several questions regarding antibody sensitivity and specificity were addressed. Is the *B. globigii* spore surface immunogenic? What is the nature and distribution of epitopes recognized by antibodies raised against spore and vegetative cell preparations? Do these antibodies cross-react with epitopes from near- and distant-neighbour *Bacillus* species? Will the presence of germinated or damaged spores influence the molecular recognition specificity of the antibody response?

Analysis of the AB-BgD antibody molecular recognition specificity (Figs 2, 3 and 4a,b) highlights the challenges in producing species-specific immunoassays. The presence of vegetative cells in the antigen preparation resulted in sera that recognized epitopes common to all four near- and distant-neighbour *Bacillus* species tested. Avoiding vegetative cell antigen contamination will clearly be of importance in generating species-specific spore antibodies. Avoiding the presence of damaged or germinated spore antigens is also of importance if the goal is to generate immunoassays that recognize the native spore surface.

The data shown in Fig. 4c,d establish that the AB-BgN polyclonal antibody preparation, raised against *B. globigii* spore and vegetative cell antigens, recognized several spore surface epitopes. These data demonstrate that the spore surface is indeed immunogenic and that antibodies can be generated against multiple spore surface proteins. The AB-BgN antibody cross-reacted with a limited set of epitopes present on the near-neighbour *B. subtilis* spore surface but did not react with the distant-neighbour *B. cereus* and *B. anthracis* spore surfaces. Results with the AB-BgN antibody further reinforce the necessity of avoiding the presence of vegetative cell antigens that can elicit non-specific antibody responses (Fig. 4d).

The data presented here suggest that it is possible to generate antibodies that recognize the *B. globigii* spore surface. The purity and uniqueness of the spore surface antigen presented to the immune system will clearly be of primary importance in developing reagents that specifically recognize spore surfaces. These conclusions are consistent with similar studies of *B. anthracis* and clostridium spore antibodies reported elsewhere (Longchamp and Leighton 1999; Quinlan and Foegeding 1997).

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DSRC DECONTAMINATION STUDY

Thursday, January 28th and Friday, January 29th

B. anthracis Spore Structure and Germination: New Targets for Decontamination

Presentation by **Terrance Leighton**

Center for Antimicrobial Discovery and Diagnostics, 401 Barker Hall, University of California, Berkeley, California 94720-3203.

ABSTRACT

The molecular genetic, biochemical and physiological basis for *Bacillus* spore formation and germination will be reviewed. Emerging opportunities for the discovery of new anthrax decontamination agents by exploiting anthrax genomic, spore structural and spore germination triggering information will be discussed. The presentation will focus on: (1) strategies for the identification and validation of spore-specific decontamination targets; (2) specific examples of new targets for anthrax decontamination agent discovery; and (3) high-throughput spore germination assays for the discovery of new germinants and antigerminants. Results from real-time multidimensional germination data analyses will be presented. These data suggest that germination chemosensing may be species-specific. High resolution soft x-ray imaging tools for the analysis of viable spore germination, and the structural biological effects of sporicides, will be described. This suite of emerging technologies affords multiple opportunities for mitigating the performance and specificity limitations of existing anthrax decontamination agents.

WHOLE-GENOME SEQUENCING OF BACILLUS ANTHRACIS

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To promote the development of target-based medical countermeasures for *Bacillus anthracis*, The Institute for Genomic Research (TIGR) has initiated an effort to sequence the genome of a virulence-plasmid cured *B. anthracis* Ames strain. The approach, used by TIGR for 10 other microbial genome projects as of July 1999, is to construct a 1.5 - 3.0 kb (small-insert) plasmid library from randomly sheared chromosomal DNA, obtain enough sequence to provide 7 – 8-fold nucleotide coverage of the genome and then assemble the data into contigs (assemblies). To 'close' gaps between assemblies, a combination of PCR techniques, small-insert clone walking and information obtained from end-sequencing large-insert libraries (*e.g.* bacteriophage λ and/or BAC) will be used.

We made a number of libraries in the pUC18 vector, either using *SmaI* blunt ends, or *BstXI* linkers, and, as of 7 June 1999, had generated 15,549 good sequencing reactions from 21,012 attempts (74% efficiency) with an average read length of 568 nt. Based on an estimated genome size of 4.25 Mb, this represented an average coverage of 2.1-fold per base. The average G + C content of this DNA sequence was 36.4%.

Assembly of the *B. anthracis* sequences to date has been in line with the Poisson distribution-based Lander-Waterman model (Genomics 1988 2: 231-239): sequences from the small-insert library have assembled into about 3794 contigs, totaling 4.2 Mb. Thus, our small-insert library is behaving as if it is highly representative of the entire *B. anthracis* genome.

A summary of our preliminary analyses of the *B. anthracis* genomic sequence will be presented.

The *B. anthracis* sequence will be made available through the TIGR microbial database site.

Contact: Dr. Eric Eisenstadt, ONR Code 335, 800 North Quincy Street, Arlington, VA 22217-5660; Tel: (703) 696-4596; Fax: (703) 696-1212

Two most suitable sessions for abstract: **Management of dangerous pathogens**; **Defining dangerous pathogens**

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